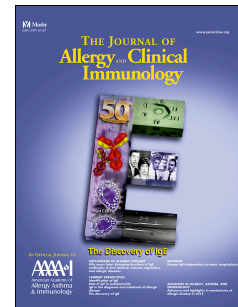


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Sialylation of IgG antibodies inhibits IgG-mediated allergic reactions

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Sialylation of IgG antibodies inhibits IgG-mediated allergic reactions

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Disclosure of potential conflict of interest:

Hansa Medical AB (HMAB) (www.hansamedical.com) holds patents for using EndoS as a treatment for antibody-mediated diseases. M.C. is listed as one of the inventors on these applications and has a royalty agreement with HMAB. Genovis AB (GAB) (www.genovis.com) holds patents for the biotechnological use of EndoS where M.C. is listed as an inventor. HMAB and GAB were not involved in any way in the design of the study, writing of the manuscript or the decision to publish.

The rest of the authors declare that they have no relevant conflicts of interest and no financial interests.

79 **Key words:**

80 Adjuvant; allergy; allergen-specific immunotherapy; anaphylaxis; antibody; birch
81 pollen allergy; galactose; IgE; IgG; IgG Fc glycosylation; sialic acid; SignR1.

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Capsule Summary:

In presence of high allergen dosis besides IgE also IgG antibodies can induce allergic reactions, whose severity is dependent on the induced type of IgG Fc glycosylation, what should be considered for new AIT protocols containing new adjuvants.

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To the Editor,

IgE antibodies (Abs) can mediate allergic reactions, including systemic anaphylaxis by activating the high affinity FcεRI on mast cells and basophils, leading to release of inflammatory mediators^{1,2,E1-3} (see References E1-45 in this article's Online Repository). In contrast, allergen-specific IgG Abs, which are also induced by allergen-specific immunotherapies (AIT), can inhibit IgE-mediated anaphylaxis caused by low levels of allergen through allergen-masking and crosslinking of the FcεRI with the classical IgG inhibitory receptor FcγRIIb^{2,3,E1,4,5}. However, when allergen levels are high, IgG Abs induced in untreated and SIT-treated allergic patients as well as to medical drugs also have the potential to mediate anaphylaxis by activating classical activating FcγRs on different immune cell types^{2,3,4,E6-12}.

The effector functions of IgG Abs depend on their subclass^{E11,13} and the type of Fc N-glycosylation (**Fig 1, A**, and **Fig E1**). Agalactosylated IgG Abs generally promote inflammation^{E14,15}, whereas galactosylation and terminal sialylation of IgG Abs generally suppress inflammation^{5-8,E15-19}. However, the effects of IgG subclass and Fc glycosylation pattern in allergy remain unclear.

Here, we first compared the capacity of differently glycosylated forms of murine IgG1, a subclass that resembles AIT-induced human IgG4 in its limited ability to activate complement and classical activating FcγRs^{3,E1,5,11,13}, to inhibit IgE-mediated systemic anaphylaxis (**Fig 1, B-E**, and **Fig E1** (see Figs E1-4 and Methods in this article's Online Repository)). IgE-mediated anaphylaxis (assessed as decreased rectal temperature) was induced intravenously (i.v.) with 10 µg of IgE anti-2,4,6-trinitrophenyl (TNP) monoclonal Ab (mAb), followed by an i.v. challenge 24 h later with 1 µg of TNP-coupled ovalbumin (TNP-OVA) (**Fig 1, B**). Increasing doses of differently glycosylated murine IgG1 anti-TNP mAbs

(clone H5; native=low-galactosylated (low-gal), *in vitro* galactosylated (gal) or *in vitro* galactosylated plus sialylated (sialylated; sial)) decreased IgE-mediated hypothermia in a FcγRIIb-dependent manner (**Fig 1, B-E**, and **Fig E1**).

Even though low-galactosylated IgG1 showed a tendency for more efficient inhibition (**Fig 1, C**; 3 μg of IgG1; not significant), possibly due to its higher affinity than sialylated IgG1 for FcγRIIb¹⁶, the IgG glycosylation pattern (**Fig 1, D and E**) and IgG subclass (studied by comparing IgG1, IgG2a and IgG2b anti-TNP class switch variant (sv) mAbs with identical V(D)J sequences)^{E20} (**Fig E1, G** and data not shown) had only a slight effect on extent of inhibition.

In contrast, the severity of IgG-mediated systemic anaphylaxis, which required challenge with a higher antigen dose (20 μg)^{2,3}, was IgG subclass- and glycosylation-dependent (**Fig 1, G and H**, and **Fig E1**). De-sialylated plus de-galactosylated (de-gal) IgG2a and IgG2b subclass anti-TNP (sv) mAbs induced more severe anaphylaxis than de-galactosylated (sv) and low-galactosylated (H5) IgG1 mAbs (IgG2a=IgG2b>IgG1) (**Fig E1**)⁴.

IgG1-mediated anaphylaxis was inhibited by galactosylation and especially by additional sialylation (**Fig 1, G**); sialylation also significantly reduced the anaphylaxis potential of IgG2b and tended to reduce that of IgG2a (**Fig 1, G**). Sialylation even reduced the increased anaphylaxis potential of IgG1 in FcγRIIb-deficient mice⁴ (**Fig 1, H**), suggesting the importance of additional/other inhibitory mechanisms of IgG1 sialylation, such as one dependent on the C-type lectin receptor, SignR1 (**Fig 1, I**)^{7,8,E17,18}.

These observations suggest that AIT protocols that promote sialylation of human IgG4 might optimally limit the possibility of IgG-mediated systemic anaphylaxis in the presence of higher allergen doses.

138

139 To evaluate this assumption, we analyzed how conventional AIT with birch pollen
140 extract and the adjuvant aluminium hydroxide (alum) (ALK-depot SQ from ALK-Abelló)
141 affects the IgG subclass and glycosylation of anti-Bet v 1 (*Betula verrucosa* 1; the major birch
142 pollen allergen) Abs (**Fig E4**)^{9,E21-23}.

143 In untreated patients, Bet v 1-specific IgG4 titers were constantly low, while IgE but
144 also IgG1 titers increased during the pollen season (**Fig 2, A**, and **Fig E2**). In contrast, during
145 AIT, levels of Bet v 1-specific IgG1 increased in the first 12 months but decreased afterwards,
146 while Bet v 1-specific IgG4 titers persistently increased (**Fig 2, A**, and **Fig E2**)^{1,9,E21,22,24}.

147 However, the Fc glycosylation profile of Bet v 1-specific serum IgG Abs from
148 untreated and AIT-treated patients remained stable and were more highly galactosylated and
149 sialylated than IgG autoAbs from rheumatoid arthritis (RA) patients^{E14} (**Fig 2, B** and **C**, and
150 **Fig E2**). The glycosylation profiles of the AIT-treated patients resembled those of two
151 recently described AIT patients who had received similar therapy with alum (Allergovit from
152 Allergopharma)⁵ and of those in therapeutic IVIg, which have Fc sialylation-dependent anti-
153 inflammatory properties (**Fig 2, B** and **C**, and **Fig E2**)^{E16-18}.

154 Consistent with an inverse relationship between IgG sialylation and inflammatory
155 potential, we found that de-sialylation of native Bet v 1-specific IgG from the serum of AIT-
156 treated patients strongly increased its ability to activate neutrophils *in vitro* (**Fig 2, B-E**, and
157 **Fig E2**).

158 These observations suggest that conventional AIT with alum induces sialylated IgG(4)
159 Abs that probably have low potential to induce IgG-mediated allergic reactions.

160 However, studies remain required to assess how Fc glycosylation modulates the
161 effector functions of human IgG1 and IgG4 and how new AIT protocols with distinct
162 adjuvants^{1,E25-29}, will influence the human IgG subclass distribution and Fc glycosylation
163 pattern and consequently, the risk of IgG-mediated allergic reactions.

To initiate such studies, we compared the effects of enriched complete Freund's adjuvant (eCFA; highly inflammatory), alum and Monophosphoryl Lipid A (MPLA; recently approved for AIT)^{1,6,E26,27} on IgG subclass and Fc glycosylation profiles in OVA-immunized mice (**Fig 2, F, and Fig E3**). eCFA induced the highest IgG titer (eCFA>MPLA=alum) (**Fig E3, C**), but all three immunizations induced predominantly IgG1 (alum/94%>eCFA/81%>MPLA/65%) followed by IgG2b and hardly IgG2c (IgG2b+IgG2c): MPLA/35%>eCFA/19%>alum/6%) (**Fig 2, G, and Fig. E3, C**), whose functions depend on galactosylation (only IgG1) and sialylation (IgG1 and at least in part IgG2b) (**Fig 1, G**).

In contrast to only small differences in Fc glycosylation pattern between human IgG subclasses in the same sample^{30,31}, glycopeptide analysis confirmed that murine IgG2 (IgG2b and IgG2c) was, on average, much more highly galactosylated and sialylated than IgG1 (**Fig 2, H, and Fig E3**)³². Because alum and MPLA induced higher galactosylation and sialylation levels of both OVA-specific IgG1 and IgG2(b) than OVA-eCFA (**Fig 2, H**), MPLA, with the highest ratio of IgG2(b), induced the highest levels of total IgG galactosylation and sialylation as determined by HPLC glycan analysis (**Fig 2, I, and Fig E3**). Consistent, only 100 µg of purified OVA-specific IgG Abs from the OVA-eCFA group, but not from the OVA-MPLA group, induced IgG-mediated anaphylaxis (**Fig 2, J**).

Taken together, our data suggest that although IgG subclass and glycosylation pattern have relatively little effect on IgG Ab blocking of IgE-mediated anaphylaxis, increased sialylation of IgG(4) Abs should decrease the risk of IgG-induced anaphylaxis in the presence of high allergen doses. Accordingly, it seems advisable to select adjuvants for new AIT protocols^{1,E25,26} for their ability to promote sialylated IgG(4) Ab responses.

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225

Figure legends

FIG 1. Effect of differently glycosylated IgG subclass Abs on IgE- and IgG-mediated murine anaphylaxis. **A**, The conserved biantennary N-glycan (four N-acetylglucosamines (dark blue) and three mannoses (green)) at Asn 297 in the IgG Fc part can be modified by fucose (red), bisecting GlcNAc (light blue), galactose (G; yellow) and sialic acid (S; magenta). The cleavage site of *EndoS* used for IgG glycan analysis is depicted. **B**, Experimental design of IgE-mediated anaphylaxis as done in **D** and **E**. **C**, Fc glycosylation profiles of the differently glycosylated murine IgG1 anti-TNP mAbs (clone H5) that were used in the murine experiments; native=low-galactosylated (low-gal), *in vitro* galactosylated (gal) or galactosylated plus sialylated (sial). **D and E**, Inhibition of IgE-mediated temperature drop by differently glycosylated IgG1 anti-TNP mAbs (H5) in **(D)** wt and **(E)** FcγRIIb-deficient mice (in each graph: no IgG, n=8-10; low-gal, n=8-10; gal, n=5; sial, n=5); symbols represent means. One of two independent experiments is shown. **F**, Experimental design of IgG-mediated anaphylaxis as done in **G-I**. **G-I**, Decrease in body temperature induced with differently glycosylated IgG subclass anti-TNP mAbs in **(G-I)** wt or **(H)** FcγRIIb-deficient mice **(G-I)** without or **(I)** with α-SignR1 treatment; de-gal: *in vitro* de-sialylated plus de-galactosylated. **(G)** Pooled data (n=10-15) from independent experiments with n=5/group/experiment or **(H, I)** one of two independent experiments is shown.

FIG 2. Bet v 1-specific serum IgG Fc glycosylation of untreated and treated allergic patients and influence of different adjuvants on IgG Fc glycosylation. **A**, Serum titers of Bet v 1-specific IgG1 and IgG4 from untreated (season, n=8; no season, n=6 + 11 (AIT-treated, month (m) 0)) and AIT-treated (n=11) birch pollen allergic patients; black line: mean; gray: pollen season. The green data points depict the 5 AIT-treated patients who were selected

for the glycan analysis in **B** and **C** and **Fig E2**, while the red data points depict the 3 samples (patient 5 at m12 (5-12) and 5-36 and 21-18) that were chosen for *in vitro* de-sialylation and neutrophil activation in **B-E** and **Fig E2**. One of two independent ELISAs is shown. **B and C**, Percentage of (**B**) agalactosylated (G0) and (**C**) sialylated glycans from purified Bet v 1-specific IgG Abs of untreated (season, n=5; no season, n=5 + 5 (AIT-treated, m0) and the 5 selected AIT-treated patients and from IVIg and purified native and *in vitro* de-sialylated total serum IgG from patient samples 5-12, 5-36 and 21-18. The filled areas indicate the levels of agalactosylated (G0) or sialylated IgG autoAbs in RA patients for comparison^{E14}. **D and E**, Human neutrophil activation assay. (**D**) Experimental setup and (**E**) ROS production after activation with native or *in vitro* de-sialylated Bet v 1-specific IgG Abs of patient 5 (P5 m36); no IgG (black). One of two independent ROS assays is shown. **F-J**, The effect of distinct adjuvants (eCFA, alum or MPLA) on the induction of OVA-specific serum IgG Abs. **F**, Experimental design. **G**, IgG1 and IgG2 (IgG2b+IgG2c; both cannot be distinguished by glycopeptide analysis because of the comparable peptide sequence) frequencies in purified OVA-specific IgG Abs as determined by glycopeptide analysis. **H and I**, (**H**) IgG1 and IgG2 or (**I**) total IgG Fc sialylation profiles of pooled and purified OVA-specific IgG Abs as determined by (**H**) glycopeptide or (**I**) total IgG glycan analysis. **J**, IgG-mediated anaphylaxis as described in **Fig 1, F** with 100 µg of pooled and purified OVA-specific serum IgG Abs; n=4-5 per group. Symbols represent means.

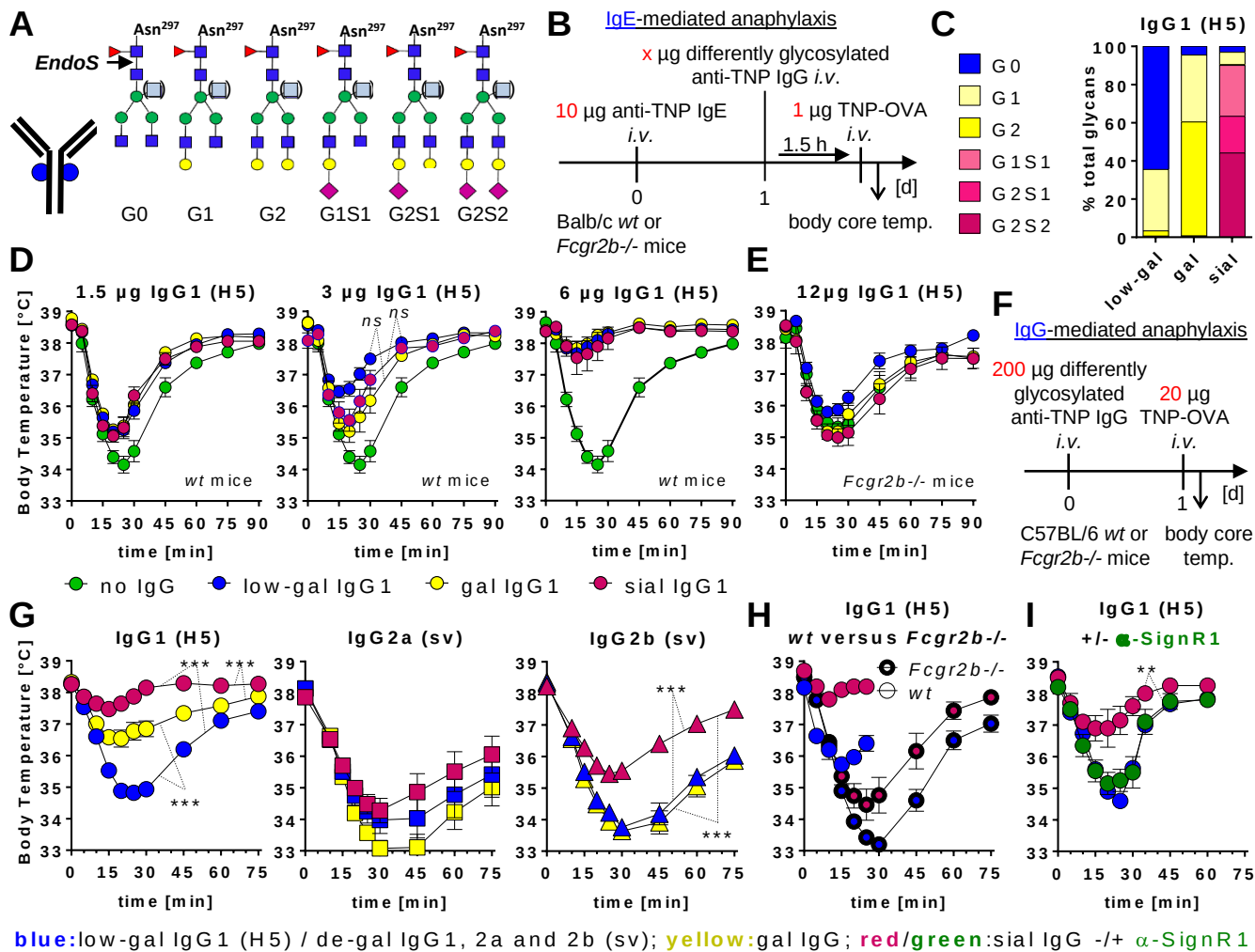


Figure 1 ↑

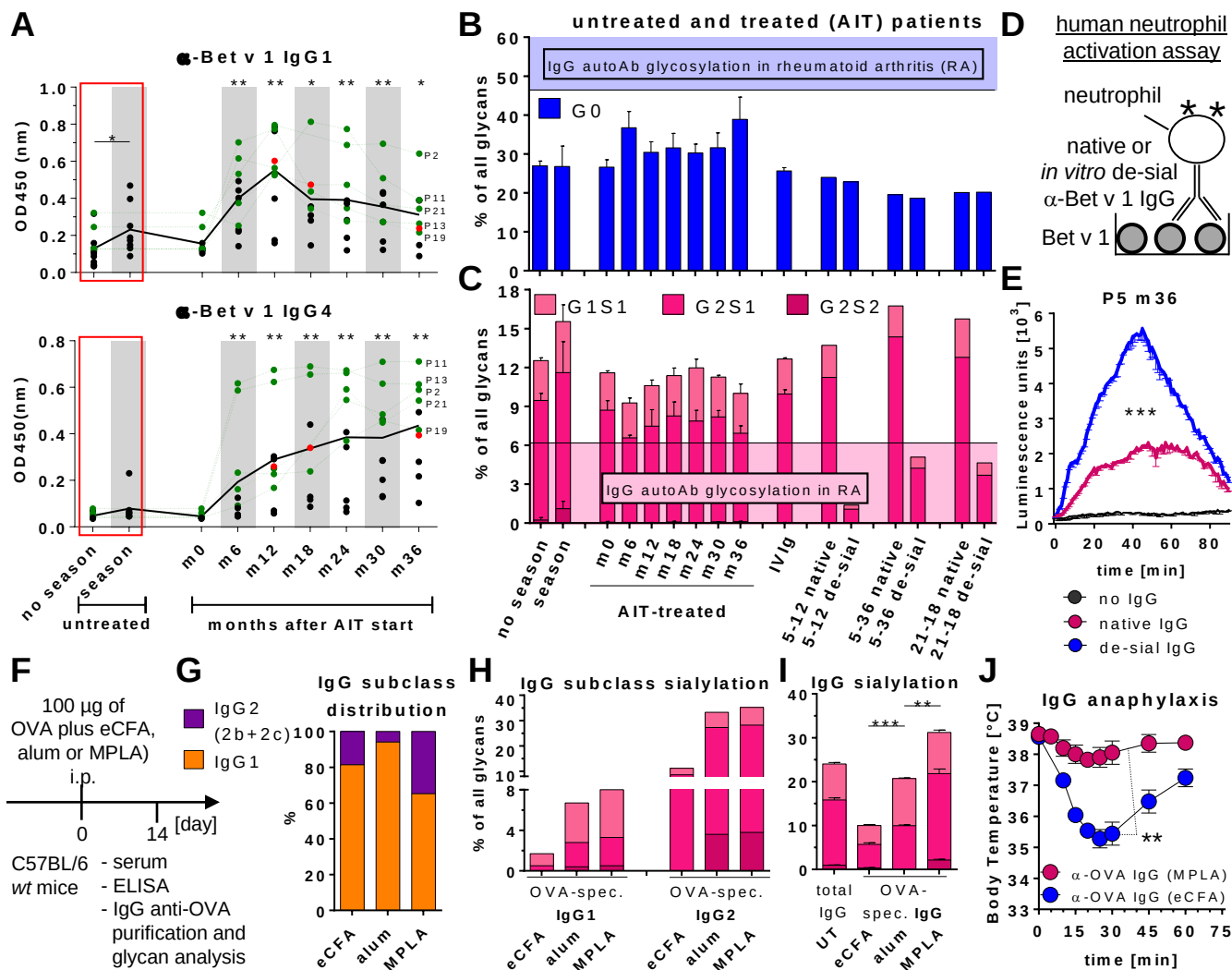


Figure 2 ↑

Supplementary Material

Supplementary Methods

Human samples

Serum samples of 7 patients (P2, P5, P7, P8, P11-13) from a previously published cohort of 15 patients^{E21-23,33} and 4 additional patients (P19-22), who completed a 3-year subcutaneous AIT with birch pollen extract containing alum (ALK-depot SQ from ALK-Abelló, Hørsholm, Denmark), were investigated. Prior to treatment, all 11 patients showed allergic symptoms to birch pollen (rhinoconjunctival symptoms +/- asthma) and were characterized by a positive skin prick test with birch pollen extract (ALK Prick SQ from ALK-Abelló), total serum IgE concentrations and serum IgE reactivity against both birch pollen extract and Bet v 1 (kUA/l; Phadia ImmunoCAP System from Thermo Fisher, Uppsala, Sweden) (**Fig 2**, and **Fig E2** and **E4**). The studies were approved by the Ethics Committee of the Medical Faculty of Philipps University, Marburg, Germany; all patients provided written informed consent to participate in the trials. Additionally, serum from untreated patients with moderate to severe birch pollen allergic symptoms was collected during (n=8) or outside (n=8) the birch pollen season (**Fig E4**) after patients provided written informed consent (Ethics Committee of the Medical Faculty of the University of Lübeck; Approval Number: 12-042). Bet v 1-specific IgE, IgG, IgG1 and IgG4 titers of untreated (season; n=8; no season: n=6) and all treated patients and the Fc glycosylation profiles of purified Bet v 1-specific IgG Abs from 10 out of the 16 untreated patients during (n=5) or outside (n=5) the birch pollen season and 5 randomly selected treated patients (P2, P11, P13, P19, P21) at different time points (**Fig 2** and **Fig E2** and **E4**) were analyzed.

Mice

All mice were bred and maintained in the specific pathogen-free (SPF) facilities at the University of Lübeck or the Cincinnati Children's Research Foundation and all experiments were done with the approval of and in accordance with regulatory guidelines and ethical standards set by the University of Lübeck and the Ministry of Schleswig-Holstein, Germany or the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center, USA, respectively. Balb/c and C57BL/6 *wild type* (wt) mice were purchased from Charles River Laboratories. *Fcgr2b*^{-/-} mice had been backcrossed for a minimum of 10 generations to the Balb/c or C57BL/6 background^{E34}. Only 8-12-week-old female mice were analyzed in the experiments. Mice were randomly assigned to groups, but a specific randomization program was not used.

Reagents

TNP-coupled BSA (TNP-BSA), BSA and TNP-OVA were purchased from Biosearch Technologies (Novato, CA, USA) and OVA from Sigma-Aldrich (St. Louis, MO, USA). Incomplete Freund's adjuvant (IFA) was purchased from Sigma-Aldrich. Enriched CFA (eCFA) was prepared by adding heat-killed *M. tuberculosis* H37 RA (DIFCO Laboratories) to IFA (5 mg *M. tuberculosis*/ml)^{E35}. Alum was purchased from Thermo-Scientific (Waltham, MA, USA), MPLA derived from *S. minnesota* R595 (MPLA-SM) from Invivogen (# vac-mpla; San Diego, CA, USA) and IVIg (Intracet; pooled serum IgG of healthy donors used in high concentrations (2 mg/kg) to treat patients with acute flares of autoimmune disease) from Biotest (Boca Raton, FL, USA).

Monoclonal Abs

The IgE anti-TNP (clone IgEL a2; ATCC-TIB-142)^{E36} and IgG1 anti-TNP (clone H5)^{E35,37} hybridoma cells and the murine IgG1, IgG2a and IgG2b anti-TNP hybridoma switch

variants (sv)^{E20} were authenticated by antigen-specific IgG subclass ELISA and grown in 0.03% Primatone RL/UF (175#DR from Kerry Biosciences, Tralee, Ireland) for Ab production. The IgG hybridoma cell lines had been tested negative for mycoplasma contamination. IgG mAbs were purified from cell culture medium with Protein G–Sepharose (GE Healthcare, Little Chalfont, UK) and IgE anti-TNP mAbs were purified using TNP-BSA coupled to CNBr-activated Sepharose 4B (GE Healthcare) prepared in our laboratory. Ab integrity was verified by SDS-PAGE, and anti-TNP reactivity was tested using ELISA. IgG Fc glycan structures were analyzed by HPLC.

Immunization and purification of OVA-specific IgG Abs.

8-10-week-old C57BL/6 mice were immunized intraperitoneally (i.p.) as indicated in **Fig 2, F**, and **Fig E3**. Serum samples were collected on day 14 and pooled OVA-specific serum IgG Abs were purified using OVA coupled to CNBr-activated Sepharose 4B (GE Healthcare) prepared in our laboratory. Enrichment of OVA-specific IgG Abs was verified using ELISA. IgG subclass distribution and Fc glycan structures were analyzed by glycopeptide analysis. The potential of the pooled and purified IgG anti-OVA Abs from distinct groups was analyzed in the IgG-mediated anaphylaxis model.

Recombinant Bet v 1

Recombinant Bet v 1-A (Bet v 1.0101; www.allergen.org) was expressed in *E. coli* BL21 (Merck Millipore, Darmstadt, Germany) cells in the form of inclusion bodies (IBs). IBs were solubilized using 6 M guanidinium hydrochloride and Bet v 1 was subsequently refolded by rapid dilution in sodium phosphate buffer pH 7.2. Folded Bet v 1 was purified by hydrophobic interaction chromatography (phenyl Sepharose 6FF high sub from GE Healthcare) and size exclusion chromatography (Superdex 75 from GE Healthcare) and finally formulated in 18 mM sodium phosphate, 135 mM sodium chloride and 10% glycerol.

78

79 **Purification of Bet v 1-specific IgG Abs**

80 Human serum IgG was purified with Protein G–Sepharose (GE Healthcare). Bet v 1-
81 specific IgG was enriched using Bet v 1 coupled to cyanogen bromide-activated Sepharose 4B
82 (GE Healthcare) prepared in our laboratory (**Fig E2**). The enrichment of Bet v 1-specific IgG
83 and exclusion of tetanus toxin-specific IgG was verified using ELISA (**Fig E2**). IgG Fc
84 glycan structures were analyzed by HPLC.

85

86 **TNP- and OVA-reactive ELISA**

87 ELISA plates were coated with 10 µg/ml of TNP-BSA or OVA +/- the indicated
88 concentrations of BSA to measure the reactivity or affinity of the indicated IgG subclass anti-
89 TNP mAbs or (purified) serum IgG anti-OVA Abs. Bound Abs were detected with
90 horseradish peroxidase (HRP)-coupled polyclonal goat anti-mouse IgG-, IgG1-, IgG2c- (the
91 isoform of IgG2a in C57BL/6 mice), IgG2b- or IgE-specific Abs purchased from Bethyl
92 Laboratories (Montgomery, TX, USA). After incubation with the 3,3',5,5'-
93 tetramethylbenzidine (TMB) substrate (BD Biosciences San Diego, CA, USA) or in **Fig E1**
94 with Femto Substrat (Thermo Fisher Scientific), optical density (OD) was measured at 450
95 nm or 425 nm, respectively.

96

97 **Bet v 1- and tetanus toxin-reactive ELISA**

98 ELISA plates were coated with 10 µg/ml of Bet v 1 or 2.5 Lf/ml tetanus toxin
99 (NIBSC, UK) to measure Bet v 1- or tetanus toxin-specific IgE, IgG, IgG1 or IgG4 levels.
100 Unless indicated otherwise, plates were incubated with 1/100 diluted serum or plasma, and the
101 bound Abs were detected with anti-human IgG (clone HP-6017, mouse IgG2a) or IgG1 (clone
102 HP-6001, mouse IgG2b) and horseradish peroxidase-conjugated polyclonal goat anti-mouse
103 IgG2a or anti-mouse IgG2b secondary Abs, respectively, horseradish peroxidase-conjugated

anti-human IgG4 (HP-6025, mouse IgG1) or horseradish peroxidase-conjugated polyclonal anti-human IgE (all antibodies from Bethyl Laboratories).

***In vitro* de-sialylation and/or de-galactosylation of IgG Abs**

The purified (native) IgG Abs were de-sialylated (de-sial) with *sialidase A* (#GK80040 from ProZyme, Hayward, CA, USA) or additionally de-galactosylated (de-gal) with $\beta(1-4)$ -galactosidase (*Streptococcus pneumoniae*) (#GKX-5014 from ProZyme). Anti-TNP and anti-Bet v 1 reactivities of differently glycosylated Abs were analyzed by ELISA. IgG Fc N-glycosylation was analyzed by HPLC.

***In vitro* galactosylation and/or sialylation of IgG Abs**

In vitro galactosylation and sialylation of purified IgG Abs was performed in a 2-step procedure, as previously described^{E16,35,38}. Briefly, purified (native) Abs were galactosylated (gal) with human $\beta 1,4$ -galactosyltransferase and UDP-galactose or subsequently additionally sialylated (sial) with human $\alpha 2,6$ -sialyltransferase and CMP-sialic acid (the substrates and transferases were purchased from Calbiochem (Nottingham, UK) or Roche (Basel, Switzerland) or the $\alpha 2,6$ -sialyltransferase was produced as described^{E39,40}. The anti-TNP reactivity of differently glycosylated Abs was tested using ELISA. IgG Fc N-glycosylation was analyzed by HPLC.

Glycan analysis of purified IgG Abs via HPLC

N-glycans were isolated from purified IgG samples by hydrolysis with recombinant *endoglycosidase S* (*EndoS*) from *Streptococcus pyogenes*^{E35,38,41}. *EndoS* cleaves the Fc N-glycans of IgG Abs at the chitobiose core between the first and second GlcNAc (**Fig 1, A**, and **Fig E1**). The resulting N-glycans were purified by solid phase extraction using home-made CarbohydrateTM graphitized carbon columns (Fisher Scientific, Hampton, NH, USA)^{E42} and

labeled with anthranilamide (2-AB; Sigma-Aldrich), as previously described^{E43}. The hydrophilic interaction liquid chromatography (HILIC)-HPLC with the labeled glycans was performed on a Dionex Ultimate 3000 (Thermo Fischer Scientific, Waltham, MA, USA) using an Xbridge XP BEH Glycan column (2.5 μ m, 100 x 4.6 mm i.d.) (Waters Corp., Milford, MA, USA). Peak identity was confirmed by analyzing the collected peak fractions using MALDI-TOF MS as described^{E35,38}. Glycans with human or murine sialic acids (human N-acetylneuraminic acid [Neu5Ac] or murine N-glycolylneuraminic acid [Neu5Gc]) had different retention times. Based on the terminal sugar moiety, the peaks were assigned to one of the following nine groups: G0 + bisecting GlcNAc, G0 - bisecting GlcNAc, G1 + bisecting GlcNAc, G1 - bisecting GlcNAc, G2 + bisecting GlcNAc, G2 - bisecting GlcNAc, G1S1, G2S1 and G2S2. Peaks containing both sialic acid and bisecting GlcNAc were not detected. The calculated proportions of the bisecting GlcNAc versions of G0, G1 and G2 were added to the percentages of the G0, G1 and G2 versions without bisecting GlcNAc, respectively, to present here six groups totaling 100%: G0, G1, G2, G1S1, G2S1 and G2S2 (**Figs 1 and 2**, and **Figs E1-3**). Mouse IgG Abs rarely have a bisecting GlcNAc, whereas 10-15% of human IgG Abs have a bisecting GlcNAc. Because more Fc glycans of total serum IgG from untreated C57BL/6 mice are sialylated than pooled serum IgG from healthy human donors (IVIg), the percentages of sialylation of murine and human IgG Abs cannot directly be compared.

IgG Fc subclass glycopeptide analysis

In contrast to only small differences in Fc glycosylation pattern between human IgG subclasses in the same sample^{E30,31}, the Fc glycosylation pattern of murine IgG subclass Abs in the same sample highly differ (de Haan et al, 2017). Accordingly, IgG Fc subclass glycopeptides were analyzed as described previously^{E44}. In short, OVA-specific IgG Abs were cleaved with trypsin, and IgG1 and IgG2 (IgG2b and IgG2c; both cannot be distinguished because of the comparable peptide sequence) Fc-glycopeptides were analyzed

by nano-liquid chromatography-mass spectrometry (LC-MS). Glycopeptide signals were assigned and quantified^{E32}, and IgG1 versus IgG2 ratios and their summarized G0, G1, G2, G1S1, G2S1 and G2S2 forms were calculated as described above. Glycans without fucose or with bisecting GlcNAc were hardly detected.

Experimental passive IgE-mediated systemic anaphylaxis.

On day 0, female mice were primed i.v. with 10 µg of IgE anti-TNP (clone IgEL a2; ATCC-TIB-142^{E36}). 22.5 h later, mice were treated with various amounts of IgG subclass anti-TNP mAbs and challenged i.v. with 1 µg of TNP-OVA 1.5 h later. Changes in the body core/rectal temperature were measured to assess the severity of systemic anaphylaxis (Physitemp BAT-12R thermometer from Science Products GmbH, Hofheim, Germany).

Experimental passive IgG-mediated systemic anaphylaxis.

200 µg of IgG subclass anti-TNP or 100 µg of purified OVA-specific IgG Abs were injected i.v. into female C57BL/6 mice. 24 h later, the mice were challenged i.v. with 20 µg of TNP-OVA. The severity of anaphylaxis was measured by determining the changes in the body core/rectal temperature.

α-SignR1 treatment

The α-SignR1 (clone 22D1) mAb was purchased from BioXCell (West Lebanon, NH, USA). 100 µg of this Ab were i.v. injected 1 h before IgG subclass anti-TNP Ab injection to induce SignR1 internalisation^{E17}.

Human neutrophil activation assay

Human neutrophils were isolated from heparinized peripheral blood from healthy adult donors as previously described^{E45}. To generate plate-bound, immobilized immune complexes,

50 µg/ml of Bet v 1 in 0.05 mM carbonate/bicarbonate buffer (pH 9.6) was coated on Lumitrac 600 96-well plates (Greiner Bio-one, Frickenhausen, Germany) for 1 h, followed by an 18 h incubation with de-sialylated or native purified serum IgG (200 µg/200µl/well). Freshly isolated neutrophils in chemiluminescence medium (RPMI medium without phenol red and sodium bicarbonate containing 20 nM HEPES and 0.06 mM Luminol; 2×10^6 cells/ml) were seeded in the plates with the immobilized immune complexes (2×10^5 cells/well). The sum of the intra- and extracellular reactive oxygen species (ROS), a sign of neutrophil activation, was measured (duplicate measurements) by luminol-amplified chemiluminescence for 1.5 h at 37°C using FluoStar Omega (BMG Labtech, Ortenberg, Germany).

Statistical analysis

The analysis of the OVA injection data and ROS data (area under the curve, AUC) were performed using Student's t-test. The body core/rectal temperature data (AUC) were analyzed using one-way ANOVA. Longitudinal analyses of the Bet v 1-specific antibody titers, prick tests and clinical scores were performed using the Wilcoxon signed-rank test. All experiments were not blinded. If not stated otherwise, data were expressed as mean values $\pm$ standard error of the mean (SEM). In all experiments normal distribution was assumed. *P < 0.05, **P < 0.01 and ***P < 0.001.

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Supplementary Figure legends

FIG E1. Generation, analysis and function of differently glycosylated IgG subclass Abs.

A, The conserved IgG biantennary N-glycan at Asn297 can be modified by fucose (red), bisecting GlcNAc (light blue), galactose (G; yellow) and sialic acid (S; magenta). **B**, Exemplary HPLC glycan peaks of *in vitro* de-sialylated plus de-galactosylated (de-gal), galactosylated (gal) or galactosylated plus sialylated (sial) IgG2a anti-TNP mAbs (sv). **C**, Fc glycosylation profiles of the differently glycosylated murine IgG1, IgG2a and IgG2b anti-TNP switch variants (sv) that were used in the murine experiments; native=low-galactosylated (low-gal). **D-F**, IgG1 anti-TNP IgG1 affinity ELISA. **(D)** Experimental setup. ELISA plates were coated with TNP-BSA alone or with increasing amounts of BSA as indicated as ratios (TNP-BSA:BSA = 1:0, 1:10 or 1:1000). **(E)** Modification of the Fc-glycan of IgG1 anti-TNP mAbs (clone H5) did not change affinity. **(F)** IgG1 anti-TNP clone H5 has a higher affinity than the IgG1 anti-TNP switch variant (sv). One out of two independent ELISA experiments is shown in **E** and **F**. **G**, IgE-mediated anaphylaxis as described in **Fig 1, B** with 10 µg of low-gal IgG subclass anti-TNP mAbs (sv) in C57BL/6 wt or FcγRIIb-deficient mice. One of two independent experiments is shown. **H**, IgG-mediated anaphylaxis as described in **Fig 1, F** and **G** with low-gal (H5) or de-gal (sv) IgG subclass anti-TNP mAbs in wt mice; each IgG subclass Ab n=10-15. Symbols represent means. Pooled data from independent experiments with n=5/group/experiment.

FIG E2. Further characterisation and Fc glycan analysis of Bet v 1-specific IgG Abs

from untreated and AIT-treated patients. A, Prick-test results and **(B)** clinical scores of the 11 AIT-treated patients (month; m). **C and D**, Serum titers of Bet v 1-specific **(C)** IgE and **(D)** IgG from untreated (season, n=8; no season, n=6 + 11 (AIT-treated, m0) and AIT-treated

(n=11) birch pollen allergic patients; black line: mean; gray: pollen season. One of two independent ELISAs is shown. The green data points depict the 5 AIT-treated patients who were selected for the glycan analysis in **Fig 2, B and C**, and **Fig E2, I and J**, while the red data points depict the 3 samples (patient 5 at m12 (5-12), 5-36 and 21-18) that were chosen for *in vitro* de-sialylation and neutrophil activation in **Fig 2, B-E** and **Fig E2, I, K and L**. **(E)** IgG4/IgG1 ratios (OD (IgG4) / OD (IgG1)) as calculated from the IgG1 and IgG4 ELISA data in **Fig 2, A. F**, Protocol for the purification and *EndoS* treatment of Bet v 1-specific serum IgG Abs. **G and H**, The amplification and purification of Bet v 1-specific IgG Abs was verified via **(G)** anti-Bet v 1 ELISA and **(H)** anti-tetanus-toxin ELISA to document the exclusion of IgG Abs that lack Bet v 1 specificity; here exemplified for patient P2 m36. **I and J**, Percentages of the different glycans (G0, G1, G2, G1S1, G2S1 and G2S2) from **(I)** summarized and **(J)** single human samples from purified Bet v 1-specific IgG Abs of untreated (season, n=5; no season, n=5 + 5 (AIT-treated, m0) and the 5 randomly selected AIT-treated patients and from IVIg and purified native and *in vitro* de-sialylated total serum IgG from patient samples 5-12, 5-36 and 21-18. **K and L**, Human neutrophil activation assay as described in **Fig 2, D and E**. **(K)** Experimental setup and **(L)** ROS production after activation with native or *in vitro* de-sialylated Bet v 1-specific IgG Abs of patient samples P5 m12 and P21 m18; no IgG (black). One of at least two independent ROS assays is shown for each patient.

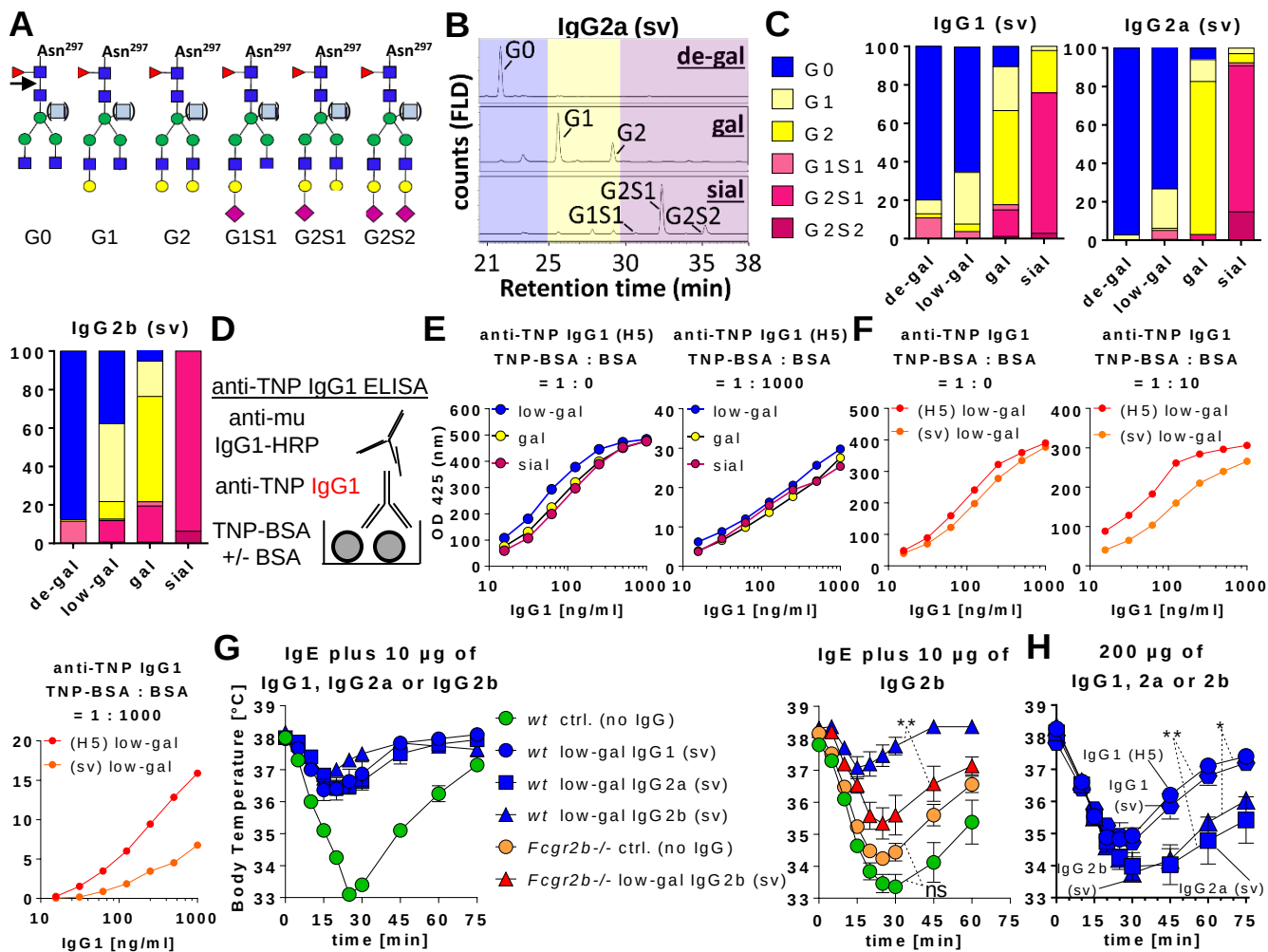
FIG E3. Different adjuvants induce distinct IgG subclass distributions and IgG Fc glycosylation pattern. **A**, Induction of differently glycosylated OVA-specific serum IgG Abs with distinct adjuvants (eCFA, alum or MPLA) as described in **Fig 2, F-J**. **B**, Design of the IgG anti-OVA Ab ELISA as used in **C**. **C**, Serum IgG (subclass) anti-OVA Ab distribution as analyzed by ELISA (n=5-10 for each group). Each ELISA represents one of two independent experiments. **D and E**, **(D)** IgG1 and IgG2 (IgG2b + IgG2c) or **(E)** total IgG Fc glycosylation

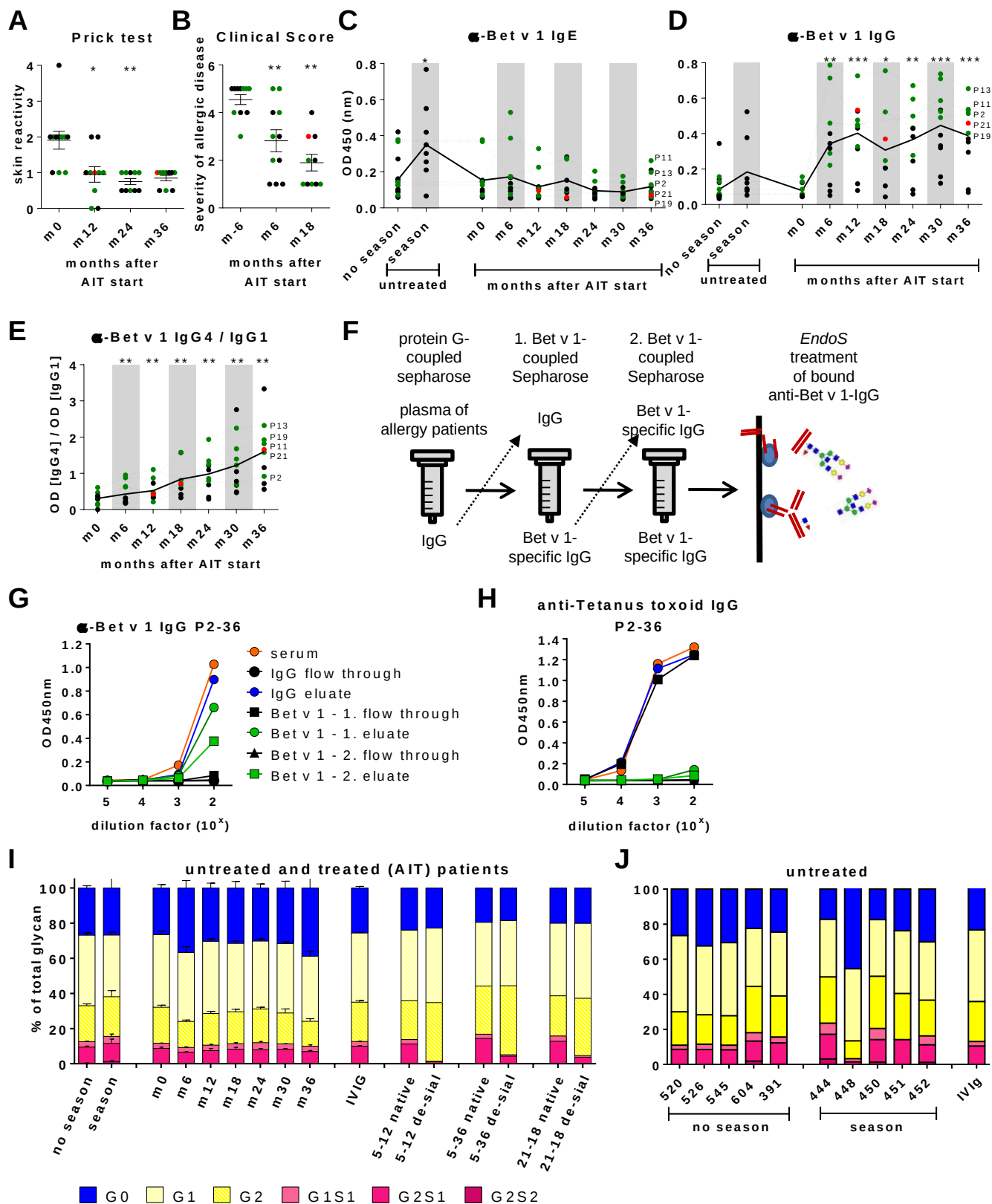
433 of all (G0, G1, G2, G1S1, G2S1 and G2S2) or only G0 forms of purified OVA-specific IgG
434 Abs as determined by (D) glycopeptide or (E) total IgG glycan analysis.

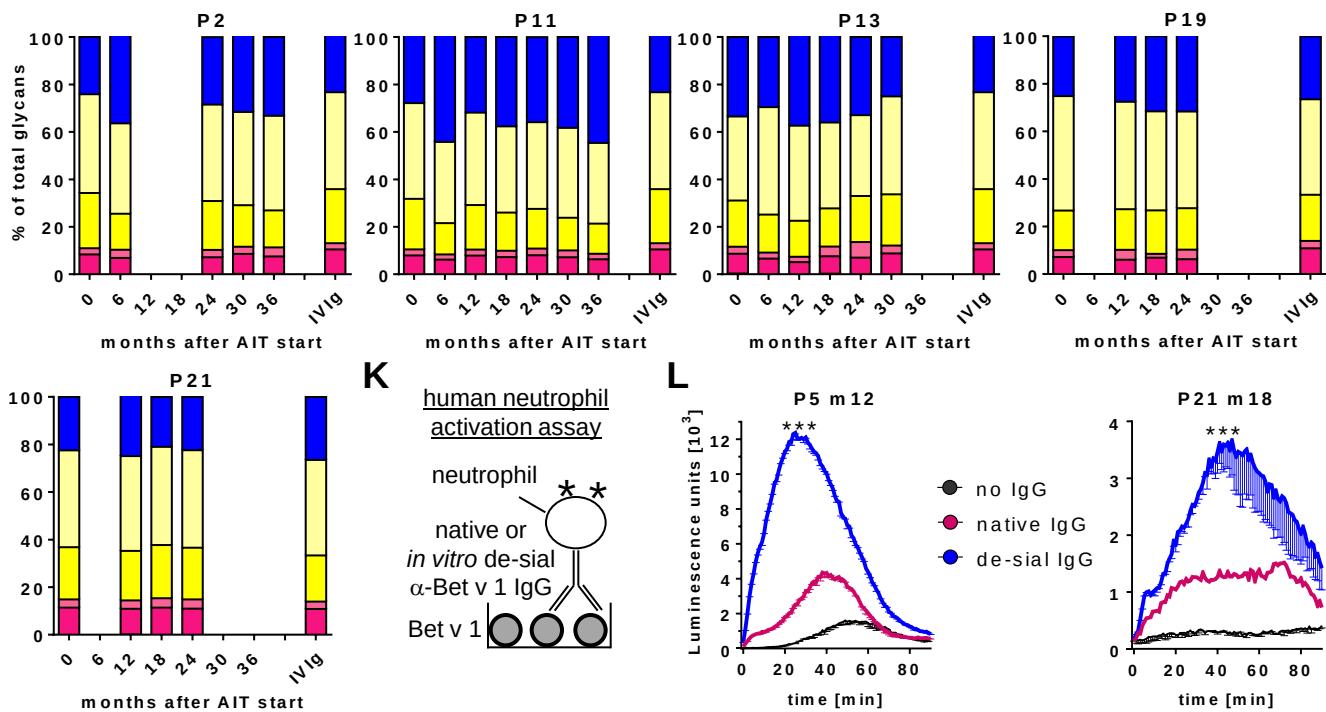
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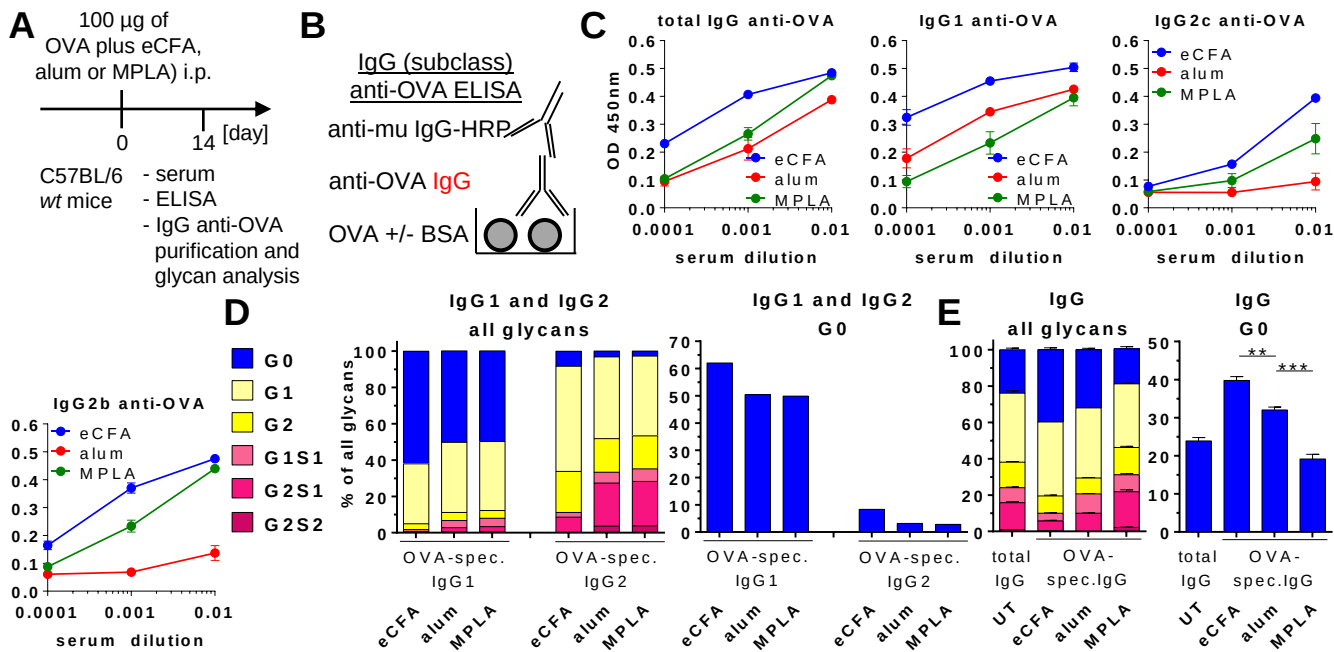
436 **FIG E4. Patients characteristics.**

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Patients characteristics

treated patients		
	n=11	Range
sex (male/female)	4/7	
age (average)	41	21-61
total IgE (kUA/l) before start of treatment, no season (average)	141	15-600
IgE against birch pollen extract (kUA/l) before start of treatment, no season (average)	9	1-41
IgE against Bet v 1 (kUA/l) before start of treatment, no season (average)	9	1-43
untreated patients, no season		
	n=8	Range
sex (m/w)	3/5	
age (average)	40	17-63
total IgE (kU/l) (average)	216	41-539
IgE against birch pollen extract (kU/l) (average)*	13	0-41
IgE against rBet v 1 (kU/l) (average)*	13	0-42
untreated patients, season		
	n=8	Range
sex (m/w)	1/7	
age (average)	45	30-56
total IgE (kU/l) (average)	141	23-269
IgE against birch pollen extract (kU/l) (average) [#]	30	0-100
IgE against rBet v 1 (kU/l) (average) [#]	33	0-100
*. [#] one patient negative for birch pollen- and Bet v 1-specific IgE showed positive prick test		